Claim 33 was also rejected under 35 USC § 112, second paragraph, as being indefinite in its recitation of the phrase "promoter controlling said DNA sequence." Claim 33 was also deemed indefinite in its recitation of "RSV" since it is allegedly unclear what term "RSV" is abbreviating or what specific RSV promoter the claim is directed to. Claim 33 was further deemed indefinite because it is allegedly unclear how the RSV promoter controls two independently recited sequences unless they are fused in-frame to one another.

Applicant appreciates the Examiner's guidance as to how to overcome the rejection of claim 33 and has taken such guidance into account in amending claims 1 and 2. Claim 1 recites that the promoter controls "expression" of said DNA sequence. Claim 2 has been amended to recite that the DNA sequence encoding the secretion signal peptide is "fused in-frame" to the DNA sequence encoding endostatin. Claim 33 has been amended in such a way as to render moot the "controls expression" and "fused in-frame" aspects of this rejection.

However, as to the "RSV promoter" aspect of this rejection, Applicant respectfully traverses and submits that one of ordinary skill in the art would readily appreciate that the term "RSV promoter" refers to the Rous sarcoma virus promoter. Included herewith are copies of relevant pages from Invitrogen's Mammalian Expression Vector brochure (available on the web at http://www.invitrogen.com/Content/World/mammalian_vectors_bro.pdf), which clearly sets forth what is meant by "RSV Promoter" (**Exhibit 1**, see particularly page 24). As evidence that the RSV promoter was well known in the art and available as of the filing date of this application, Applicant also includes herewith a copy of Mittereder *et al.*, *Hum. Gene Ther., 5:* 717-729, 1994 (**Exhibit 2**), which refers on page 718 at the bottom of the right-hand column to the RSV promoter and its availability from Invitrogen. Claim 33 has been amended to indicate that "RSV" is abbreviating "Rous sarcoma virus". Applicant respectfully submits that this does not constitute new matter because of the above-discussed knowledge in the art as to what "RSV promoter" means. Applicant therefore respectfully requests that this rejection be withdrawn.

REMARKS

The Specification

The specification has been amended to add a Sequence Listing, as well as references throughout the specification to particular sequences. A computer readable form (CRF) of the sequence listing is also enclosed. Pursuant to 37 CFR §§ 1.821-1.825, Applicant's undersigned attorney hereby states that the paper and CRF Sequence Listing are the same and contain no new matter.

The description of Figure 9 on page 5, as well as references thereto on page 29, have been amended to delete refences to "Figure 9A" and "Figure 9B" because drawings with these figure legends were not included with the application as filed. Applicant submits that such figures are not necessary for a complete understanding of the invention; therefore, Applicant respectfully requests that the application be deemed complete as filed. Because there are no Figures 9A and 9B, Figure 9C has been renamed simply "Figure 9".

The description of Figure 13 on page 6 has been amended to provide a better framework in which to include references to the sequences therein. No new matter has been added, as the added text has been copied/pasted from lines 5-9 on page 35, wherein Figure 13 is discussed.

Formal drawings are included herewith, which are believed to address all of the Draftsperson's objections set forth in the Notice Of Draftsperson's Patent Drawing Review dated 5/23/00.

The Claims

Claims 4-27 and 32 have been canceled without prejudice or disclaimer. Applicant expressly reserves the right to continue prosecuting these claims in a continuing application.

New claims 34-49 have been added. Upon entry of these new claims, the pending claims will be 1-3, 28-31, and 33-49.

Claim Objection

Claim 32 has been objected to for having a period in the middle of the claim instead of a comma. This claim has been canceled, mooting this objection.

35 USC § 112, first paragraph, Rejections

Claims 4-27 were rejected under 35 USC § 112, first paragraph, as not being enabled by the specification. Applicant traverses and submits that these claims are fully enabled by the specification. However, in the interest of advancing prosecution, Applicant has canceled claims 4-27 without prejudice or disclaimer, rendering this rejection moot. Applicant expressly reserves the right to continue prosecuting these claims and address the merits of the enablement rejection in a continuing application.

Claim 32 was rejected under 35 USC § 112, first paragraph, on the grounds that the specification does not enable vectors comprising DNA sequences lacking promoters. As to claim 32, this rejection is moot because claim 32 has been canceled. However, Applicant appreciates the Examiner raising this issue and has accordingly amended claim 1 to recite that the expression of the DNA sequence encoding endostatin is controlled by an operatively linked promoter. Applicant therefore respectfully submits that this rejection should be withdrawn and not applied to any of the pending claims.

35 USC § 112, second paragraph, Rejections

Claims 4-28 were rejected under 35 USC § 112, second paragraph, as being incomplete for omitting essential steps. The Office Action of 5/24/00 stated that the omitted step is: the mere administration of the adenovirus does not lead to expression such that the final step is commensurate with the preamble which recites "a method of expression..." As to canceled claims 4-27, this rejection is moot. As to claim 28, Applicant has amended it to positively recite in the body of the claim that "administration of the vector results in expression of endostatin in the cell." Accordingly, Applicant respectfully submits that claim 28 is now in compliance with 35 USC § 112, second paragraph, and requests that this rejection be withdrawn.

35 U.S.C. § 102 Rejection

Claims 1, 2, 4-7, 11-17, 21-24, and 28-29 were rejected under 35 U.S.C. § 102(a) as being anticipated by Leboulch *et al.* (WO 99/26480, published June 3, 1999). Applicants respectfully traverse and submit herewith a Declaration under 37 C.F.R. § 1.131 of the inventors, Paul Hallenbeck and Cheauyun Theresa Chen, swearing behind WO 99/26480. This § 1.131 Declaration, including Exhibits A-C, demonstrates that the claimed invention was conceived and reduced to practice prior to the June 3, 1999, publication date of WO 99/26480. Accordingly, Applicant respectfully submits that WO 99/26480 cannot properly be used as a § 102 reference, and requests that this rejection be withdrawn.

35 U.S.C. § 103 Rejections

Claims 1-3 were rejected under 35 U.S.C. § 103(a) as being unpatentable over Leboulch *et al.* (WO 99/26480) taken with Blezinger *et al.* Applicants respectfully traverse and submit that in view of the § 1.131 Declaration, the combination of Leboulch *et al.* (WO 99/26480) and Blezinger *et al.* cannot properly be used to reject any of Applicant's claims. Applicant therefore respectfully requests that the § 103(a) rejection of claims 1-3 be withdrawn.

Claims 32-33 were rejected under 35 U.S.C. § 103(a) as being unpatentable over Leboulch *et al.* (WO 99/26480) and Blezinger *et al.* as applied to claims 1-3, and further in view of Lemarchand *et al.* Applicants respectfully traverse and submit that in view of the § 1.131 Declaration, the combination of Leboulch *et al.* (WO 99/26480), Blezinger *et al.* and Lemarchand *et al.* cannot properly be used to reject claims 32-33. Applicant therefore respectfully requests that this rejection be withdrawn.

Claims 4, 7-10, 14, 18-21, and 25-27 were rejected under 35 U.S.C. § 103(a) as being unpatentable over Leboulch *et al.* (WO 99/26480) and Blezinger *et al.* as applied to claims 1-3, and further in view of O'Reilly *et al.* (U.S. Patent No. 5,854,205). Applicants respectfully traverse and submit that in view of the § 1.131 Declaration, the combination of Leboulch *et al.* (WO 99/26480), Blezinger *et al.* and O'Reilly *et al.* cannot properly be used to reject claims 4, 7-10, 14, 18-21, and 25-27. Applicant therefore respectfully requests that this rejection be withdrawn.

Claims 28-31 were rejected under 35 U.S.C. § 103(a) as being unpatentable over Leboulch *et al.* (WO 99/26480) and Blezinger *et al.* as applied to claims 1-3, and further in view of Kovesdi *et al.* (U.S. Patent No. 5,851,806). Applicants respectfully traverse and submit that in view of the § 1.131 Declaration, the combination of Leboulch *et al.* (WO 99/26480), Blezinger *et al.* and Kovesdi *et al.* cannot properly be used to reject claims 28-31. Applicant therefore respectfully requests that this rejection be withdrawn.

New Claims

New dependent claims 34-49 have been added. All of these claims depend directly or indirectly from either claim 1 or claim 28, so it is believed that the addition of these claims raises no new issues of patentability. Therefore, entry of these claims is respectfully requested.

New claim 34 depends from claim 29 and specifies that the recited cell is a human cell. This claim finds support *e.g.* in the paragraph spanning pages 34-35 and in Figure 13.

New claim 35 depends from claim 28 and specifies that the recited cell is an endothelial cell. This claim finds support *e.g.* in the first paragraph of page 13.

New claim 36 depends from claim 35 and specifies that the recited cell is a blood vessel endothelial cell. This claim finds support in the first paragraph of page 13.

New claim 37 depends from claim 28 and specifies that the adenoviral vector is administered to the cell *in vitro*. This claim finds support in the first paragraph of page 16.

New claim 38 depends from claim 1 and specifies that the recited promoter is an adenoviral promoter. This claim finds support *e.g.* on page 8, lines 6-7.

New claim 39 depends from claim 1 and specifies that the recited promoter is a foreign promoter. This claim finds support *e.g.* on page 8, lines 6-7.

New claim 40 depends from claim 1 and specifies that the recited DNA sequence encodes murine endostatin. This claim finds support *e.g.* on page 23 and in Figures 1B and 2.

New claim 41 depends from claim 1 and specifies that the recited DNA sequence encodes human endostatin. This claim finds support *e.g.* on page 34 and in Figures 12A and 12B.

New claim 42 depends from claim 2 and specifies that the recited secretion signal peptide is the human basement membrane protein BM40 leader. This claim finds support *e.g.* on pages 31-32 and 34 and in Figures 12A and 12B.

New claim 43 depends from claim 3 and specifies that the recited vector comprises SEQ ID NO:2. This claim finds support *e.g.* on page 23 and in Figure 1B.

New claim 44 depends from claim 42 and specifies that the recited vector comprises SEQ ID NO:5. This claim finds support *e.g.* on page 34 and in Figure 12B.

New claim 45 depends from claim 1 and specifies that the recited vector is free of at least the majority of adenoviral E1 and E3 DNA sequences. This claim finds support on page 7, line 14.

New claim 46 depends from claim 45 and specifies that the recited vector is also free of at least a portion of at least one DNA sequence selected from the group consisting of adenoviral E2 and E4 DNA sequences. This claim finds support on page 7, lines 17-18.

New claim 47 depends from claim 1 and specifies that the recited vector is free of all or a portion of each of the adenoviral E1 and E4 DNA sequences. This claim finds support on page 9, lines 20-21.

New claim 48 depends from claim 1 and specifies that the recited vector is free of all or a portion of each of the adenoviral E1 and E2 DNA sequences. This claim finds support on page 9, lines 21-22.

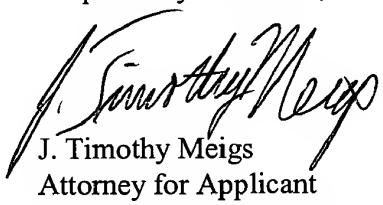
New claim 49 depends from claim 1 and specifies that the recited vector is free of all or a portion of each of the adenoviral E1, E2, and E4 DNA sequences. This claim finds support on page 9, lines 22-23.

Conclusion

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached pages are captioned "**Version With Markings To Show Changes Made.**"

In view of the above amendments and remarks and attached Declaration of the inventors under 37 C.F.R. § 1.131, it is submitted that this application is now ready for allowance. Early notice to this effect is solicited. If in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject application, the Examiner is invited to call the undersigned attorney.

Respectfully submitted,



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Date: November 22, 2002

Version With Markings To Show Changes Made

In the specification

The description of Figure 1B at page 3, lines 17-18, has been replaced with the following re-written figure description:

-- Figure 1B shows the DNA sequence encoding and the amino acid sequence of murine endostatin, to which is attached a mouse Ig-K leader sequence (SEQ ID NO:1 and SEQ ID NO:2); --

The description of Figure 5 at page 4, lines 12-26, has been replaced with the following re-written figure description:

-- Figure 5 shows adenoviral-mediated expression and secretion of murine endostatin in S8 cells. The mEndo (lane 2) and NullI (lane 3) supernatant proteins were analyzed by SDS-PAGE. Each 60 μg supernatant protein was analyzed on 4 to 12% linear gradient pre-casted gel. The protein standard marker was run on lane 1. The gel was stained with Gelcode Blue stain reagent to visualize the protein bands. As indicated, the expected murine endostatin protein band around 20 Kd (marked by arrow) was generated only from Av3mEndo but not from the control Av3NullI. After being transferred to a PVDF membrane from a duplicate SDS-PAGE, the 20 Kd protein band was excised from an immoblin membrane blot and subjected to N-terminal protein sequencing analysis. The determined protein sequence (SEQ ID NO:3) is shown at the bottom with arrows marked as the beginning of the N-termini of two major secreted proteins, 80% containing additional amino acid residues of DAA, and 20% containing residue A from murine Ig-K signal peptide. The results demonstrated that S8 cells transduced with Av3mEndo expressed and secreted murine endostatin after it was processed from murine Ig-K signal peptide; --

The description of Figure 9 at page 5, lines 18-27, has been replaced with the following re-written figure description:

-- Figure 9 depicts the results of a B16F10 mouse lung metastasis study. C57BL/6J mice were treated with Av3mEndo (n=20), Av3NullI (n=20), or HBSS (n=12) by tail vein injection at

$2x10^{11}$ particles per mouse. Two days later, lung metastasis was established by tail vein injection of B16F10 cells at $5x10^4$ cells per mouse. 14 days after tumor implantation, the study was ended. [(Figure 9A)] Blood levels of murine endostatin were determined. [Each bar represents the blood level of mEndo from an individual animal. (Figure 9B)] Surface lung metastasis was determined. [Each open circle represents lung metastasis from an individual animal. The bar represents the average lung metastasis of each group with different treatment. (Figure 9C).] Liver transduction (copy number per hepatocyte) was correlated to blood endostatin levels. --

The description of Figure 12B at page 6, lines 12-14, has been replaced with the following re-written figure description:

-- Figure 12B shows the DNA sequence encoding human endostatin, and the amino acid sequence of human endostatin, to which is attached a human BM40 basement membrane protein leader sequence (SEQ ID NO:4 and SEQ ID NO:5). --

The description of Figure 13 at page 6, lines 15-16, has been replaced with the following re-written figure description:

-- Figure 13 shows adenoviral-mediated expression and secretion of human endostatin in S8 cells. The determined protein sequence (SEQ ID NO:6) is shown at the bottom of Figure 13 with arrows marked at the beginning of the N-termini of three major secreted proteins, 50% containing the additional amino acid residues APQQEALA (SEQ ID NO:7), 25% containing residues LA, and 25% containing no residues from human BM40 basement protein signal peptide. --

The paragraph at page 17, lines 9-18, has been replaced with the following re-written paragraph:

-- The mouse endostatin cDNA was PCR amplified from mouse collagen XVIII clone ID #748987 from GenomeSystems, Inc. with the primers of SEQ ID NO:8 and SEQ ID NO:9: 5'-ACT GGT GAC GCG GCC CAT ACT CAT CAG GAC TTT CAG CC-3' and 5'-AAG GGC TAT CGA TCT AGC TGG CAG AGG CCT AT-3' (598 bp F1 fragment). The mouse Ig-kappa leader was PCR amplified from pSecTag2 (Invitrogen) with the primers of SEQ ID NO:10 and

SEQ ID NO:11: 5'-CAC TGC TTA CTG GCT TAT CG-3' and 5'-CTG ATG AGT ATG GGC CGC ACC AGT GG-3' (147 bp F2 fragment). PCR was carried out with Pfu DNA polymerase (Stratagene) for 35 cycles in the following conditions: 95°C hot start for 3 min., 95°C denaturation for 1 min, 55°C annealing for 1 min, and 72°C extension for 2 min. The DNA fragments were gel purified. --

The paragraph at page 17, lines 19-27, has been replaced with the following re-written paragraph:

-- The sig-mEndo chimeric DNA (718 bp) was generated by PCR splice overlap extension (Horton, *et al.*, Biotechniques, Vol. 8, pgs. 528-535 (1990)) with F1 and F2 DNA fragments generated above as templates to assemble mouse Ig-kappa leader sequence and murine endostatin cDNA. PCR was carried out with the primers of SEQ ID NO:8 and SEQ ID NO:12: 5'-ACT GGT GAC GCG GCC CAT ACT CAT CAG GAC TTT CAG CC-3' and 5'-CTG ATG AGT ATG GGC CGC GTC ACC AGT GG-3' using Pfu DNA polymerse (Stratagene, LaJolla, California). PCR was run for 35 cycles in the following conditions: 95°C hot start for 3 min., 95°C denaturation for 1 min, 60°C annealing for 1 min, and 72°C extension for 2 min. --

The paragraph at page 23, lines 3-15, has been replaced with the following re-written paragraph:

-- Murine endostatin cDNA was PCR generated from the C-terminus of mouse α1(XVIII) collagen clone ID #748987 from GenomeSystems. The cDNA was assembled with murine Ig-kappa leader to generate sig-mEndo chimeric for the secretion of murine endostatin protein by PCR splice overlap extension (Horton, *et al.*, 1990). The sig-mEndo chimeric DNA was cloned into the Nhe1 and ClaI sites of the adenoviral shuttle plasmid, pAvF91xr to create pAvmEndoLxr (Fig. 1A). The entire sig-mEndo chimeric sequence was confirmed by auto sequencing analysis. The consensus sequence (SEQ ID NO:1) and derived protein sequence (SEQ ID NO:2) are shown in Fig 1B. The adenoviral vector encoding sig-mEndo chimeric was generated by the "Quick Cre/Lox 2 plasmid system" in 293 cells by transient transfection with pcmvE2a, pCre, pSQ3 and pAv3mEndoLx through Cre/Lox mediated recombination. The

generated vector was RCA negative. The correct genome structure of generated Av3mEndo vector was confirmed by restriction digests and Southern Blot analysis (Fig. 3A and B). --

The paragraph at page 29, lines 4-13, has been replaced with the following re-written paragraph:

-- The B16F10 lung metastasis model was established in C57B16/J mice by tail vein injection of 5×10^4 cells per mouse. Two days before tumor implantation, the mice were treated with Av3mEndo vector by tail vein injection at 2×10^{11} particles per mouse. Controls were carried out with either equal volume of HBSS or the equal amount of Av3NullI vector. Fourteen days post tumor implantation, blood was collected from all animals and analyzed by mEndo ELISA. [As shown in Fig.9A, the] The mice treated with Av3mEndo vectors all demonstrated higher level of murine endostatin at an average of 708 ± 435 ng/ml. In contrast, the control mice treated with HBSS and Av3NullI only showed the endogenous level of murine endostatin at an average of 50 ± 15 and 56 ± 19 ng/ml, respectively. --

The paragraph extending from page 29, line 19, to page 30, line 5, has been replaced with the following re-written paragraph:

-- Lung surface metastasis was determined in all mice. [As shown in Fig.9B, the] The control mice treated with HBSS showed the highest number of lung metastases at an average of 109 ± 65 number of lung metastases/mouse. Mice treated with Av3mEndo vectors demonstrated the reduction of lung metastases to the average of 36 ± 18 number of lung metastases/mouse (33% relative to HBSS control). However, mice treated with Av3NullI vectors also demonstrated the reduction of lung metastases to the average of 42 ± 28 number of lung metastases/mouse (39% relative HBSS control). This indicated that the majority of anti-metastasis effect (61%) was caused by Av3 backbone vector and some reduction (6%) was caused by murine endostatin secretion. The current study does prove that Av3mEndo expressed and secreted functional murine endostatin was demonstrated in vitro. Systemic administration of Av3mEndo demonstrated sustained blood level of endostatin as demonstrated on day 16 post vector injection. The results support that anti-angiogenesis gene therapy of angiogenic inhibitor gene delivery may provide a means to reduce lung metastasis. --

The paragraph extending from page 31, line 21, to page 32, line 13, has been replaced with the following re-written paragraph:

-- The human endostatin cDNA was PCR amplified from the cDNA of human α1 (XVIII) collagen. The human liver cDNA was generated from human liver poly A RNA (Clonetech, Palo Alto, CA) by reverse transcriptase polymerase chain reaction (RT- PCR). The reverse transcription was carried out with the primer of 5'-TTT TTT TTT CAG TGT AAA AGG TC-3' (SEQ ID NO:13) using the Perkin Elmer RT-PCR kit (Perkin Elmer Applied Biosystems, Foster City, CA) for 1 cycle in the following conditions: room temperature for 10 min, 42°C reverse transcribing for 3 min, 99°C denaturation for 5 min, 5°C cooling for 5 min, and hold at 4°C until the cDNA was ethanol precipitated and resuspended. The 790 bp human endostatin cDNA fragment was PCR amplified from the prepared cDNA with the primers of SEQ ID NO:14 and SEQ ID NO:15: 5'-CAG ATG ACA TCC TGG CCA G-3' and 5'-CTA TAC AGG AAA GTA TGG CAG C-3'. PCR was carried out for 35 cycles in the following condition: 95°C hot start for 3 min, 80°C for 3 min followed by the addition of Pfu DNA polymerase (Stratagene, La Jolla, CA), 95°C denaturation for 1 min, 55°C annealing for 1 min, and 72°C extension for 3 min. The 790 bp human endostatin cDNA fragment was gel purified and reamplified as described except using the annealing temperature of 58°C. The 790 bp human endostatin cDNA fragment was gel purified and cloned into PCR-Script Amp SK^+ using PCR-Script Cloning Kits (Stratagene) according to the manufacturer's procedure to generate pcrhend 1. The human endostatin cDNA region of the pcrhend 1 plasmid was confirmed with the direct sequencing analysis by Gene Therapy Core Technologies Molecular Core Laboratory at Genetic Therapy, Inc. Gaithersburg, MD. --

The paragraph extending from page 32, line 14, to page 33, line 4, has been replaced with the following re-written paragraph:

-- The human endostatin cDNA fragment was assembled with human BM40 basement protein leader according to the following procedure. The BM40 basement protein leader was generated by annealing 2 pieces of synthesized oligonucleotides, 5'-GCC AAG CTT CCA TGA GGG CCT GGA TCT TCT TTC TCC TTT GCC TGG CCG GGA GGG CTC TGG CAG CCC

CTC AGC AAG AAG CGC TCG CTC ACA GCC ACC GCG ACT TCC AGC CGG TGC TCC A-3' (sense; SEQ ID NO:16), and 5'-CCA GGT GGA GCA CCG GCT GGA AGT CGC GGT GGC TGT GAG CGA GCG CTT CTT GCT GAG GGG CTG CCA GAG CCC TCC CGG CCA GGC AAA GGA GAA AGA AGA TCC AGG CCC TCA TGG AAG CTT GGC-3' (antisense; SEQ ID NO:17) followed by Hind III and Sex A1 digestion. Th digested BM40 basement protein leader was cloned into Hind III and Sex A1 sites of pcrhend 1 to generate pBmpcrhen plasmid. The entire sig-hEndo region of the pBmpcrhen plasmid was confirmed with the direct sequencing analysis by Gene Therapy Core Technologies Molecular Core Laboratory at Genetic Therapy, Inc. The adenovial shuttle plasmid pAV1bmhend1x was generated by substitution of the Factor IX (F9) containing sequence with the sig-Endo containing sequence in pAvF9Lxr adenoviral shuttle plasmid in the following procedure. An 800 bp fragment containing sig-hEndo sequence was generated from pBmpcrhen by SacI digestion followed by Klenow fill in and Sal I digestion. The pAvF9Lxr plasmid was digested with Bam HI restriction enzyme followed by Klenow fill in and digested with Sal I restriction enzyme to remove F9 containing sequences. The two digested fragments were gel purified and ligated to generate pAV1bmhendlx. (Figure 12A). --

The paragraph at page 34, lines 8-19, has been replaced with the following re-written paragraph:

-- Human endostatin cDNA was RT-PCR generated from the C-terminus of cDNA of human α1 (XVIII) collagen from human liver poly A RNA. The human BM40 basement protein leader was generated from two pieces of synthesized oligonucleotides. The annealed human BM40 basement protein leader was cloned 5' of the human endostatin cDNA to generate sig-hEndo chimeric protein for the secretion of human endostatin protein. The sig-hEndo chimeric DNA was cloned into the adenoviral shuttle plasmid, pAvF9lxr to create pAV1bmhendlx (Fig. 12A). The entire sig-hEndo chimeric sequence was confirmed by auto sequencing analysis. The consensus sequence (SEQ ID NO:4) and derived protein sequence (SEQ ID NO:5) are shown in Fig 12B. The adenoviral vector encoding sig-hEndo chimeric was generated by the "Quick Cre/Lox 2 plasmid system" in S8 cells by transient transfection with pCre, pSQ3, and pAV1bmhendlx through Cre/Lox mediated recombination. --

The paragraph extending from page 34, line 21, to page 35, line 12, has been replaced with the following re-written paragraph:

-- Av3bmhendlx mediated human endostatin expression and secretion was characterized in vector transduced S8 cells. As shown in Figure 13, the supernatant protein of Av3bmhendlx, i.e., human endostatin, was analyzed by SDS-PAGE (lanes 1-8 and 10). Each 20 μg of supernatant protein was analyzed on 4 to 12% linear gradient precasted gel. The protein standard marker was run on lane 9. The SDS-PAGE was transferred to a polyvinylidene fluoride membrane. The membrane was stained with Coomassie blue R-250. The 20 kDa protein bands, corresponding to the correct size of human endostatin, were excised from a membrane blot and subjected to N-terminal protein sequencing analysis. The determined protein sequence (SEQ ID NO:6) is shown at the bottom of Figure 13 with arrows marked at the beginning of the N-termini of three major secreted proteins, 50% containing the additional amino acid residues APQQEALA (SEQ ID NO:7), 25% containing residues LA, and 25% containing no residues from human BM40 basement protein signal peptide. The 20 kDa protein was not shown in the supernatant protein from Av3NulI cells. (Figure 5). The results demonstrated that S8 cells transduced with Av3bmhendlx expressed and secreted human endostatin after it was processed from human BM40 basement protein signal peptide. --

The enclosed Sequence Listing, pages 1-8, has been inserted into the specification.

In the claims

Claims 4-27 and 32 have been canceled without prejudice or disclaimer. New claims 34-49 have been added. Claims 1, 2, 28, and 33 have been amended as shown below. Also shown below are the unamended pending claims. Therefore, the following is the complete set of claims that will be pending upon entry of the instant amendment.

1. (Amended) An adenoviral vector [including] comprising a DNA sequence encoding endostatin operatively linked to a promoter controlling expression of said DNA sequence.

2. (Amended) The vector of Claim 1, [and] further comprising a DNA sequence encoding a secretion signal peptide immediately 5' and fused in-frame to said DNA sequence encoding endostatin.

3. The vector of Claim 2 wherein said secretion signal peptide is the secretion signal peptide of Ig-Kappa.

28. (Amended) A method of expressing endostatin in a cell, comprising:

administering to a cell the adenoviral vector of Claim 1, whereby administration of the vector results in expression of endostatin in the cell.

29. The method of Claim 28 wherein said cell is a mammalian cell.

30. The method of Claim 29 wherein said cell is an A549 cell.

31. The method of Claim 29 wherein said cell is a Hep3B cell.

33. (Amended) The vector of Claim [32, and further comprising an] 1, wherein said promoter is a Rous sarcoma virus (RSV) promoter [controlling said DNA sequence encoding endostatin and said DNA sequence encoding the secretion signal peptide of Ig-Kappa].

34. (New) The method of Claim 29 wherein said cell is a human cell.

35. (New) The method of Claim 28, wherein said cell is an endothelial cell.

36. (New) The method of Claim 35, wherein said cell is a blood vessel endothelial cell.

37. (New) The method of Claim 28, wherein the adenoviral vector is administered to the cell *in vitro*.

38. (New) The vector of Claim 1, wherein said promoter is an adenoviral promoter.

39. (New) The vector of Claim 1, wherein said promoter is a foreign promoter.

40. (New) The vector of Claim 1, wherein said DNA sequence encodes murine endostatin.

41. (New) The vector of Claim 1, wherein said DNA sequence encodes human endostatin.

42. (New) The vector of Claim 2, wherein said secretion signal peptide is the human basement membrane protein BM40 leader.

43. (New) The vector of Claim 3, comprising SEQ ID NO:2.

44. (New) The vector of Claim 42, comprising SEQ ID NO:5.

45. (New) The vector of Claim 1, wherein the vector is free of at least the majority of adenoviral E1 and E3 DNA sequences.

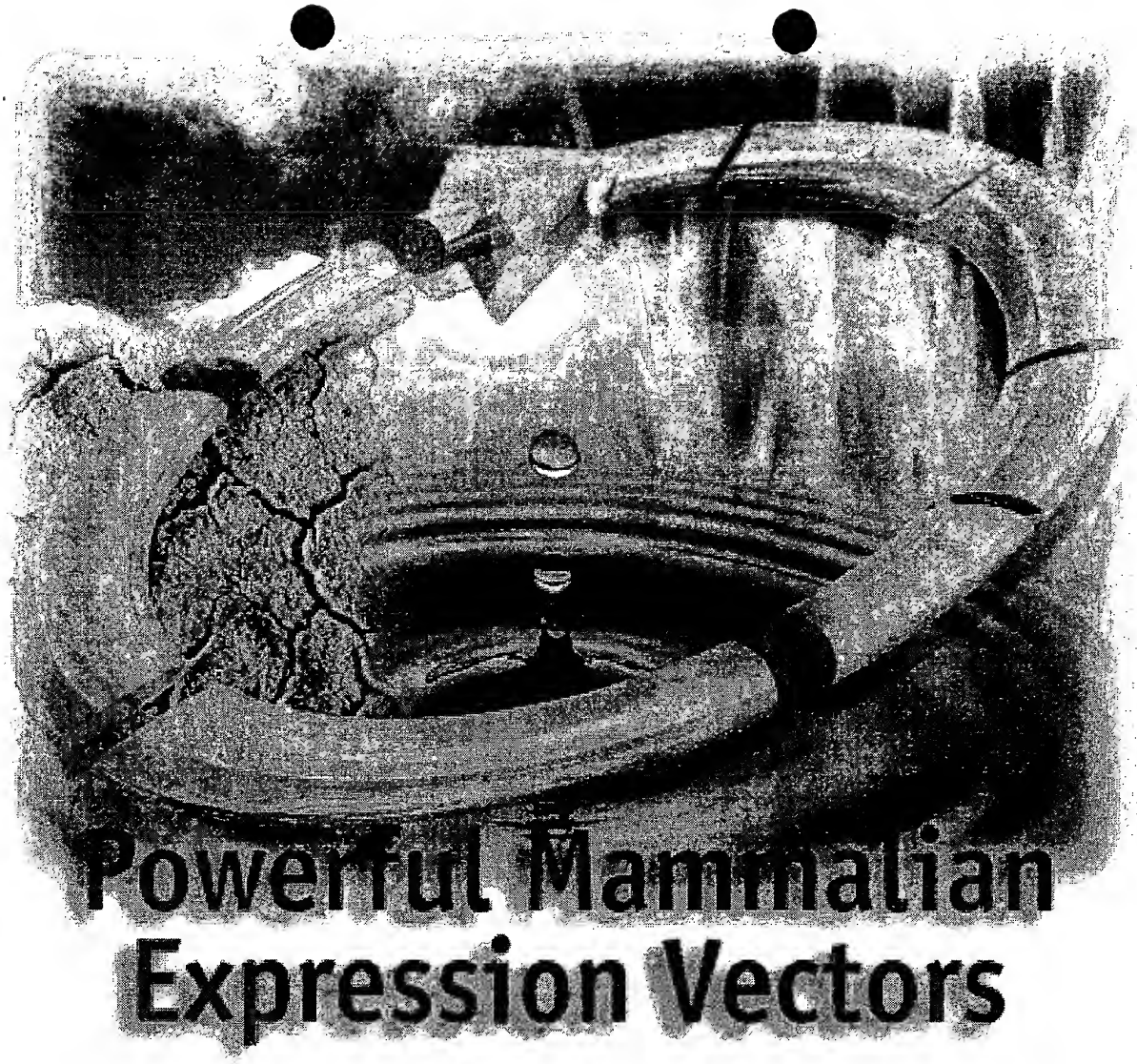
46. (New) The vector of Claim 45, wherein the vector is also free of at least a portion of at least one DNA sequence selected from the group consisting of adenoviral E2 and E4 DNA sequences.

47. (New) The vector of Claim 1, wherein the vector is free of all or a portion of each of the adenoviral E1 and E4 DNA sequences.

48. (New) The vector of Claim 1, wherein the vector is free of all or a portion of each of the adenoviral E1 and E2 DNA sequences.

49. (New) The vector of Claim 1, wherein the vector is free of all or a portion of each of the adenoviral E1, E2, and E4 DNA sequences.

EXHIBIT 1



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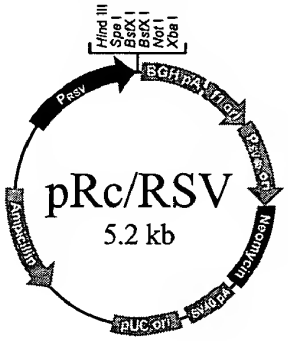
Finding the right mammalian expression vector can have a significant impact on the success of your experiment. By learning about the basic elements involved in expressing recombinant protein in mammalian cells, you can make a well-informed decision. This brochure contains a wealth of information about the large selection of mammalian vectors available from Invitrogen. To make it easier to navigate, it has been divided into two sections. For general information on the promoters, selectable markers, and fusion tags available in Invitrogen's mammalian expression vectors, or for assistance choosing the right vector for your experiment, start with Section 1, pages 2-8. For details on a particular mammalian expression vector, check out the vector-specific information in Section 2, pages 9-24.

High-level RSV Expression from the RSV Promoter.

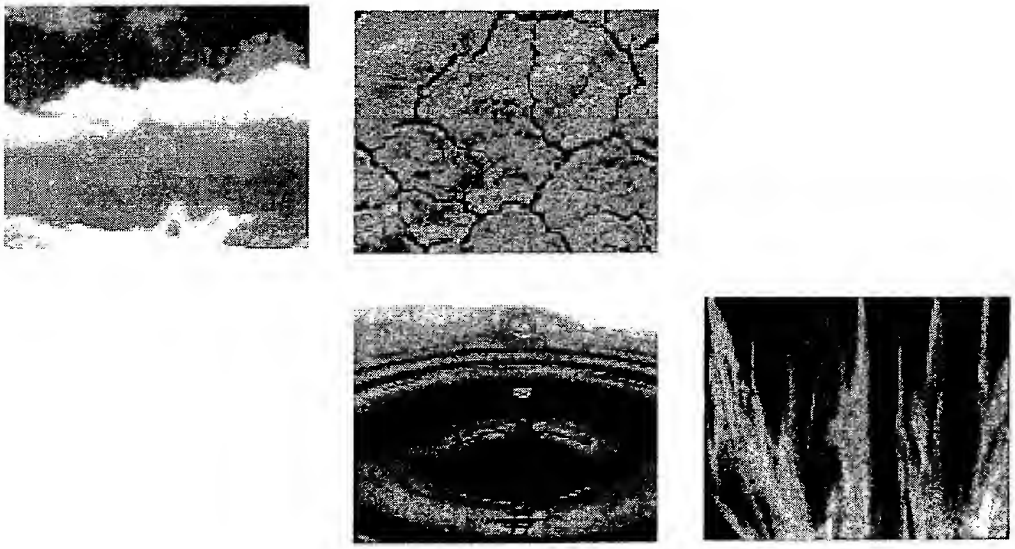
The pRc/RSV uses the Rous sarcoma virus (RSV) enhancer-promoter for high-level expression of recombinant proteins in mammalian cells. The RSV promoter has been shown to work particularly well in avian and murine cell lines (30). In addition, this vector offers the following features:

- Bovine growth hormone (BGH) polyadenylation signal to enhance mRNA stability
- SV40 origin for transient episomal replication and simple vector rescue in cell lines expressing the SV40 large T antigen (i.e. COS-1 and COS-7)
- Neomycin Selection marker for generating stable mammalian cell lines
- Ampicillin resistance gene for selection in *E. coli*
- pUC origin for high-copy maintenance in *E. coli*



Vector	Cat. no.
pRc/RSV	V780-20

pRc/RSV uses the RSV promoter for high-level expression in mammalian cells.



References:
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EXHIBIT 2

HUMAN GENE THERAPY 5:717–729 (1994)
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Evaluation of the Efficacy and Safety of *In Vitro*, Adenovirus-Mediated Transfer of the Human Cystic Fibrosis Transmembrane Conductance Regulator cDNA

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ABSTRACT

Cystic fibrosis (CF) is a common, fatal recessive disease caused by mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) gene manifested by abnormalities in the regulation of chloride ion (Cl^-) secretion across the apical membrane of epithelial cells throughout the body. Adenovirus-mediated delivery of the normal CFTR cDNA and correction of the CF epithelial cell Cl^- secretory phenotype suggests the feasibility of gene therapy for CF lung disease. Few studies, however, have focused on the evaluation of the safety of the adenovirus-mediated gene transfer approach. This study presents *in vitro* data on the efficacy and safety of adenovirus-mediated transfer of the human CFTR cDNA using Av1Cf2. Av1Cf2-mediated transfer of the human CFTR cDNA complemented the abnormal cAMP-regulated Cl^- permeability of cells with the CF epithelial phenotype. Av1 vectors did not replicate infectious virus in HeLa cells infected *in vitro*, although trace vector DNA synthesis was observed at very high multiplicity of infection. Expression of the adenoviral late gene for the hexon capsid protein was observed at trace levels in Av1 vector-infected HeLa cells, but not in freshly isolated human bronchial epithelial cells, consistent with the pattern of DNA synthesis observed in these different target cells. Altogether, these observations support the efficacy and safety of use of Av1Cf2 for treatment of the fatal pulmonary component of CF.

OVERVIEW SUMMARY

Transfer of the normal human cystic fibrosis transmembrane conductance regulator cDNA to the human airway using the recombinant, E1-deleted, E3-deleted adenoviral vector AV1Cf2 has been proposed for the treatment of the fatal pulmonary component of CF (Wilmott *et al.*, 1993). The present study provides data supporting the biological efficacy and safety of Av1Cf2-mediated gene transfer *in vitro*.

INTRODUCTION

CYSTIC FIBROSIS (CF) is the most common lethal autosomal recessive disease in the United States (Boat, 1989), with an incidence in the Caucasian population of between 1 in 2,000 and 1 in 2,500. The major clinical manifestations of CF are in the gastrointestinal tract and in the lungs, although more than 95% of CF patients eventually die of the pulmonary sequelae, with a median survival age of 29.4 years (Cystic Fibrosis Patient Registry, 1991, Annual Report). New hope for gene

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therapy for this disorder has come from a series of recent events, including elucidation of the pathophysiology of chloride ion (Cl^-) transport in CF epithelial cells (Frizzell *et al.*, 1986; Li *et al.*, 1988; Hwang *et al.*, 1989); identification and cloning of the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which when abnormal, results in CF (Kerem *et al.*, 1989; Riordan *et al.*, 1989; Rommens *et al.*, 1989) as well as the molecular analysis of CFTR mutations (Collins, 1992; Tsui, 1992).

Human gene transfer and therapy approaches for other genetic diseases have been underway using vectors derived from murine retroviruses since early 1989 (Anderson, 1992; Miller, 1992). The feasibility of *in vivo* gene therapy for lung disease in CF patients is supported by *in vitro* studies showing that the cAMP-stimulated Cl^- permeability defect in CF epithelial cells could be corrected by transfer of the normal human CFTR cDNA using vaccinia (Rich *et al.*, 1990) or retroviral vectors (Drumm *et al.*, 1990). However, at this time neither of these vectors can be considered seriously for clinical use to correct the Cl^- defect in airway epithelial cells for reasons of toxicity or efficacy of gene transfer, respectively. In regard to the latter issue, retroviral gene transfer requires an actively dividing cell target for gene transfer to occur; however, the pulmonary epithelium is not a rapidly dividing tissue, making the use of retroviral vectors unlikely to be effective in this organ. In contrast, another vector system derived from human adenovirus (Stratford-Perricaudet *et al.*, 1992), which readily infects the nondividing lung epithelium is being evaluated by several groups in preclinical and clinical studies of CF gene therapy (Crystal, 1992; Welsh, 1992; Wilson, 1992; Boucher and Knowles, 1993; Wilmott *et al.*, 1993). Animal studies with these vectors have previously demonstrated expression of human α1 antitrypsin (Rosenfeld *et al.*, 1991) and human CFTR (Rosenfeld *et al.*, 1992; Rich *et al.*, 1993) in the bronchial epithelium of the cotton rat. Further, several of the National Institutes of Health Recombinant DNA Advisory Committee (NIH RAC)-approved human clinical trials of adenovirus-mediated CFTR gene transfer have commenced, and correction of the chloride ion transport defect in the nasal epithelium after *in vivo* gene transfer has been reported (Zabner *et al.*, 1993).

Clinical use of adenovirus vectors for human gene therapy to the lung is attractive due to the high efficiency of gene transfer that can be achieved with them, the fact that nonreplicating cells are easily infected, and that the wild-type adenovirus from which the vectors are derived naturally infects human airway epithelial cells (Ginsberg, 1984). However, wild-type, replication-competent adenovirus is a respiratory pathogen that commonly causes a mild upper respiratory tract infection (*e.g.*, the common cold; Straus, 1984; Horwitz, 1990a). Therefore, even though the adenoviral vectors proposed for current clinical trials have been disabled in an effort to deactivate replication, a careful evaluation of the safety was the principal focus in preclinical studies and also in the first round of clinical trials in humans.

This study reports the preclinical, *in vitro* safety and efficacy characterization carried out for an adenoviral vector, Av1Cf2, recently approved by the NIH RAC for use in a human clinical trial to transfer the human CFTR cDNA into respiratory epithelium of the nose and lungs of individuals with CF (Wilmott *et al.*, 1993; Trapnell, 1993). A companion paper (Yei *et al.*, 1994) describes the *in vivo* animal safety studies performed with the same vector.

METHODS

Cells

293 cells, a human embryonic kidney epithelial cell line transformed with the left-hand 11% of the Ad5 genome, were obtained at passage 31 (American Type Culture Collection [ATCC] CRL 1573; Graham *et al.*, 1977) and maintained in improved minimal essential medium (IMEM; Bio-Whittaker) containing 10% fetal bovine serum (FBS), 1 m*M* glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (IMEM-10). CFPAC, a pancreatic adenocarcinoma cell line from a CF individual homozygous for the ΔF508 CFTR allele, was obtained from R. Frizzell (University of Alabama) (Schoumacher *et al.*, 1990) and maintained in Dulbecco's modified Eagle's medium (DMEM, Bio-Whittaker) supplemented as above (DMEM-10). MLE-12 is a murine lung epithelial cell line derived from mice bearing a transgene consisting of the surfactant protein C promoter driving expression of the SV40 large T antigen (Bachurski *et al.*, 1993; Wikenheiser *et al.*, 1993). This cell line exhibits the CF epithelial cell Cl^- secretory phenotype, *e.g.*, absence of cAMP (forskolin)-stimulated Cl^- efflux. MLE-12 cells were maintained as described previously (Wikenheiser *et al.*, 1993). HeLa cells (ATCC [CCL2]), an epithelial-like cell line derived from a cervical carcinoma, was maintained as for CFPAC. Human bronchial epithelial (HBE) cells were obtained at bronchoscopy from the mainstem bronchi with a cytology brush (Microinvasive, Inc. Watertown, MA), as previously described (Trapnell *et al.*, 1991a). Informed consent was obtained from all individuals prior to bronchoscopy and brushing. HBE cells were removed from the cytology brush by shaking into DMEM and HBE cell explants, maintained in DMEM-10, and used without delay.

Adenoviral vectors: construction, propagation, and titration

Replication-competent, serotype 5 adenovirus (wild-type; Ad5), and Ad-*dl*327, an Ad5-derived mutant harboring a deletion of *Xba* I fragment D (78.5–84.7 map units [mu]) were obtained from Thomas Shenk (Department of Molecular Biology, Princeton University). Adenoviruses and recombinant adenoviral vectors were propagated, purified, and titered using 293 cells as previously described (Graham and Van Der Eb, 1973; Graham *et al.*, 1977; Rosenfeld *et al.*, 1992).

Recombinant, replication-deficient adenoviral vectors were constructed by the homologous recombination method (Berkner, 1988) using pAvS6, an adenoviral vector construction "shuttle" plasmid and Ad-*dl*327 (Trapnell, 1993). pAvS6 is a pSKII$^-$ (Stratagene)-based plasmid containing the following contiguous elements: (i) Ad5 sequences 1–392 (Genbank accession no. M73260; containing the left inverted terminal repeat [ITR], encapsidation signals (Ψ), and the E1a enhancer), an artificial *Asc* I site, the Rous sarcoma virus (RSV) long terminal repeat promoter (sequences 209–605 from the plasmid pRC/RSV, Invitrogen), an artificial *Sfi* I site, a spliced form of the

Ad5 major late mRNA tripartite leader (obtained by reverse-transcription polymerase chain reaction amplification of total RNA from Ad5-infected 293 cells) inserted as a *Not* I–Xba I fragment; (ii) the SV40 early polyadenylation site (sequences 880–1,013 from the plasmid pSVSPORT1; Bethesda Research Labs) inserted as a blunt fragment into the *Sal* I site of pSKII$^-$; and (iii) an Ad5 sequence used for homologous recombination (Ad5 sequences 3,328–6,241) inserted as a blunt fragment into the *Xho* I site with base 3,328 oriented closest to the SV40 polyadenylation site. AvICf2, an E1-deleted (1.18 mu to 9.2 mu), E3-deleted (78.5 to 84.7 mu) adenoviral vector was constructed by first inserting the normal human CFTR cDNA coding sequence fragment into the *Eco* RV site of pAvS6 so that the 5′ end of the CFTR coding sequence was closest to the Ad5 TPL. The CFTR cDNA was removed as a *Pst* I fragment from the plasmid pBQ4.7 (provided by L.-C. Tsui, the Hospital for Sick Children, Toronto, Canada) and inserted as a blunt fragment. The expected nucleotide sequence of the pAvS6-CFTR plasmid was confirmed by automated DNA sequencing (E. Otto, M. Kaloss, Genetic Therapy, Inc., unpublished observations) and the plasmid was linearized with *Kpn* I and recombined with the large (35 kb) *Cla* I fragment of Ad-*dl*327 in 293 cells as described (Rosenfeld *et al.*, 1992; Trapnell, 1993). After double-plaque purification, the identity of the clonal isolates was confirmed by Southern analysis, immunohistochemical staining for CFTR expression in AvICf2-infected cells, and immunoprecipitation of CFTR, as previously described (Rosenfeld *et al.*, 1992; Tolstoshev *et al.*, 1993).

AvICf1 was constructed in a similar manner using a shuttle plasmid, pAvS2, containing the Ad5 ITR, Ψ, E1a-enhancer, and human CFTR cDNA, but utilizing the Ad5 major late promoter (MLP) instead of the RSV promoter, and the endogenous E1b polyadenylation signal instead of the SV40 sequence in the heterologous minigene. All other elements are the same as in AvICf2. AvICf1 has been previously evaluated in separate preclinical studies (Crystal *et al.*, 1992). AvINull1 is identical to AvICf1, except that it contains no structural gene coding sequence in the heterologous minigene.

Evaluation of AvICf2-mediated correction of the CF epithelial cell phenotype

CFPAC or MLE cells were grown on membrane supports (Millipore) as described (Schoumacher *et al.*, 1990) and evaluated for the presence of cAMP (forskolin)-stimulated Cl^- conductance essentially as described (Welsh, 1985). When cells had reached confluence and a stable transepithelial resistance, cells were infected with AvICf2 at a multiplicity of infection (moi) of 50 infectious units (iu)/cell. Based on previous studies (Rosenfeld *et al.*, 1992), this moi was expected to target a high percentage of cells sufficient to complement the phenotype of the CFPAC cells. Twenty four hours later, the membranes were placed in a modified Ussing chamber (Analytic Bioinstrumentation Department, Case Western Reserve, Cleveland) in media consisting of: 2 mg/ml D-glucose, 143.5 m*M* Na^+, 5 m*M* HPO_4^{2-}, 1 m*M* $H_2PO_4^-$, 1.2 m*M* Mg^{2+}, 1.2 m*M* SO_4^{2-}, 1 m*M* Ca^{2+}, and either 139.8 m*M* Cl^- (Cl^--containing media) or 139.8 m*M* SCN, 1 m*M* calcium acetate (instead of $CaCl_2$) and balanced osmotically with sucrose (for Cl^--free media). Cells were maintained at pH 7.4, 37°C and perfused with oxygenated media on both sides of the membrane. Electrical measurements were made with a 742C voltage clamp device (Bioengineering Department, Iowa University). Forskolin (7-β-diacetyl-7-β-[γ-*N*-methylpiperazino]-butyryl, dihydrochloride, 37 μ*M*, Calbiochem) or ionomycin (a calcium ionophore, 20 μ*M*, Calbiochem) were added to increase intracellular cAMP or Ca^{2+}, respectively.

Because CFPAC cells do not easily form tight junctions, which are required for Ussing chamber work, we also evaluated complementation of the CF epithelial cell Cl^- defect using a fluorescence assay to evaluate the efficacy of AvICf2 (Wilmott *et al.*, 1993) essentially as described (Verkman *et al.*, 1990). Fluorescence studies were carried out with 6-methoxy-*N*-3-sulfopropyl guinolinium (SPQ) in CFPAC cells 24 hr after infection by AvICf2 at an moi value of 50, essentially as described (Verkman *et al.*, 1990), but using a perfusion cell designed for monolayers on glass coverslips in a front-faced fluorimeter.

Evaluation of the potential for replication of adenoviral vectors

The potential for replication and production of infectious AvI vectors was evaluated by infecting HeLa cells and then measuring the production of infectious virions by plaque assay on 293 cells. HeLa cells were grown to 70–80% confluence, infected with AvICf1, AvINull1, or Ad-*dl*327 (as a replication-competent positive control) at an moi of 60 iu/cell in IMEM-2 for 90 min with rocking, then supplemented with IMEM-10 and incubated in a 5% CO_2, humidified atmosphere. At various subsequent times, cells plus supernatant were collected, intracellular virus was released by freeze-thaw cycles (×3) and evaluated for the presence of infectious virus by plaque titration on 293 cells (Graham and Van Der Eb, 1973).

The potential for AvI vectors to replicate their DNA genome was evaluated in HeLa cells as previously described (Berkner and Sharp, 1983). Briefly, HeLa cells at 70–80% confluence were infected with replication-deficient AvICf1 or replication-competent Ad5 or both at various moi values. After 20 hr, the media was changed to phosphate-free media supplemented with [^{32}P]orthophosphate (8,500–9,120 Ci/mmol, 50 μCi/ml), incubated (5% CO_2, humidified atmosphere, 37°C) for 20 hr and then low-molecular-weight DNA (*e.g.*, viral DNA) was extracted (Hirt, 1967). Purified DNA was cleaved with *Eco* RI and subjected to electrophoresis and autoradiography. This method for evaluating viral replication is sensitive to a level of detection of 3 iu of wild-type, replication competent adenovirus per cell.

Evaluation of protein synthesis in adenoviral vector-infected cells

The potential for AvI vectors to alter the genetic program of the infected host cell was evaluated by examining the pattern of protein synthesis in infected HeLa cells and HBE cells. HeLa cells, grown to 70–80% confluence were infected with Ad5, as a positive control, AvICf1, or AvICf2 at an moi of 50 iu/cell. After 24 hr, the cells were washed twice in phosphate-buffered saline (PBS) and labeled for 24 hr in methionine-free DMEM-10 containing [^{35}S]methionine (100 μCi/ml, 1,000 Ci/

mmol). Cell lysates were prepared in RIPA buffer supplemented with protease inhibitors as previously described (Rosenfeld *et al.*, 1992). Incorporation of radioactivity into nascent protein was determined in aliquots of the cell lysate by trichloroacetic acid (TCA) precipitation and equal amounts of radioactively labeled total (cellular and viral) protein from each sample were evaluated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography of the dried gel. In these experiments, equal numbers of cells were labeled at the same specific activity, and TCA analysis indicated that incorporation of radioactivity into nascent polypeptides was similar from sample to sample except for wild-type infections where the total protein synthesis was slightly reduced. For example, in experiment 1, TCA precipitation of 10-μl aliquots of labeled samples gave: uninfected, 30,988 cpm; Av1Cf1, 32,291 cpm; and Ad5, 20,041 cpm. Because equal amounts of labeled protein of similar specific activities were evaluated, the number of cells represented in different lanes were also similar and varied by no more than two- to three-fold.

Similar studies were also carried out using freshly isolated HBE cells, the actual target cells currently proposed for gene therapy in the CF lung. Further, to evaluate the potential for delayed effects from these Av1 vectors, infected HBE cells were evaluated over the course of 7 days. HBE cells were suspended in DMEM-2 and infected with Av1Cf1 at an approximate moi of 50 iu/cell in polypropylene tubes in a 5% CO_2, humidified atmosphere at 37°C. As a positive control, cells were infected with Ad5 at a similar moi. After 90 min, cells were divided into equal aliquots and cultured as above in DMEM-10 for 1, 3, 4, or 7 days. Cells were then collected, labeled with [^{35}S]methionine for 24 hr and evaluated by SDS-PAGE as described above.

Northern analysis of adenovirus hexon gene mRNA

To evaluate whether adenoviral late genes could be expressed from Av1 vectors, HeLa cells, or 293 cells (as a positive control) were infected with Av1Cf1, Av1Null1, or Ad-*dl*327 at a moi of 100 iu/cell in DMEM-2 for 90 min, supplemented with DMEM-10, and cultured in a 5% CO_2, humidified atmosphere at 37°C. After 4 days, total RNA was extracted (Chirgwin *et al.*, 1979) and evaluated by Northern blotting as previously described (Trapnell *et al.*, 1991b) using a 400-bp ^{32}P-labeled Ad5 hexon DNA probe. Autoradiography was performed at both short (0.5 hr) and long (24 hr) exposure times to detect abundant and rare hexon mRNA expression, respectively.

Immunoprecipitation of adenovirus hexon protein

Expression of the adenoviral hexon capsid protein was evaluated in HeLa cells maintained and infected with Av1Cf1 or Ad5 (as a positive control) at an moi of 100 iu/cell as described for hexon mRNA evaluations. At various times after infection from 1 to 7 days, cells were washed twice with cold PBS and labeled with [^{35}S]methionine (1,000 Ci/mmol, 100 μCi/ml) for 24 hr. Cells were then washed twice in PBS and extracted in RIPA buffer containing antiproteases as previously described (Rosenfeld *et al.*, 1992). Cell lysate was cleared of debris by brief, low-speed centrifugation and TCA-precipitable radioactivity was determined. Equal amounts (50,000 cpm of TCA-precipitable radioactivity/lane) of TCA-precipitable radioactivity were subjected to immunoprecipitation using an anti-human adenovirus hexon mouse monoclonal antibody (generously provided by Bartels, Inc.) and evaluated by SDS-PAGE and autoradiography.

Similar studies were carried out in freshly isolated HBE cells infected and maintained as above except that cell cultures were performed in suspension in polypropylene tubes (Falcon, 2059 tubes) to minimize the manipulation and preserve the integrity of the cells. Media was changed at the appropriate times after collecting the cells by low-speed centrifugation. Three days after infection of HBE cells with Av1Cf1 or Ad5, cells were labeled with [^{35}S]methionine (1,000 Ci/mmol, 500 μCi/ml) for 24 hr, lysed, and evaluated for TCA-precipitable radioactivity, and subjected to gel electrophoresis and autoradiography as above. As a further positive control, similar evaluations were carried out using 293 cells.

RESULTS

Construction of recombinant, E1-deleted, E3-deleted adenoviral vectors

Av1Cf1 and Av1Cf2 are structurally similar recombinant, replication-deficient adenoviral vectors derived from a serotype

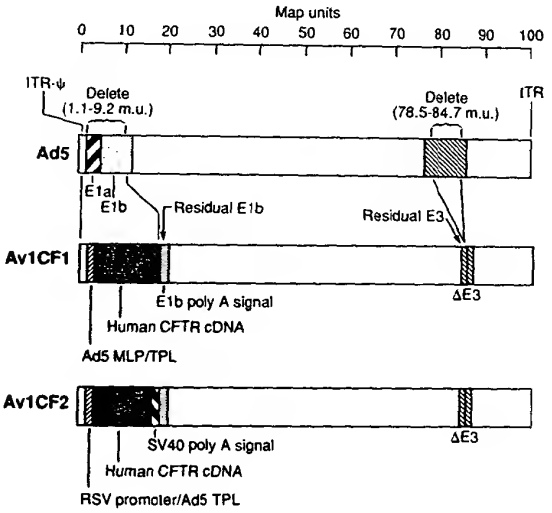

FIG. 1. Similarity in genomic organization of recombinant, replication-deficient adenoviral vectors used to transfer the human CFTR cDNA. Shown (from top) are type 5 adenovirus (Ad5), and two adenoviral vectors expressing the human CFTR cDNA (Av1Cf1, Av1Cf2). The genomic structures of each are identical except for the heterologous minigene within the E1 region deletion as noted. The vectors contain the same E1 region deletion (from 1.1 to 9.2 mu), and E3 region deletion (from 78.5 to 84.7 mu). The components of each minigene are listed below each vector genome (RSV, Rous sarcoma virus; MLP, major late promoter from adenovirus serotype 5; TPL, tripartite leader from adenovirus serotype 5; SV40, simian virus 40).

5 adenoviral genome backbone (Fig. 1) by homologous recombination of shuttle plasmids with Ad-*dl*327 (Berkner, 1988; Trapnell, 1993). The entirety of E1a and the majority of E1b genes (see Fig. 2 of Trapnell, 1993) were replaced by a heterologous minigene containing the human 4.5-kb CFTR cDNA coding sequence under control of the Ad5 major late promoter (Av1CF1) or RSV (Av1CF2) promoters. A major E3 deletion was utilized to reduce the final recombinant genome size to a value 101% of the wild-type Ad5 genome to minimize the potential for recombination and deletion. The exact nature of the E3 deletion was confirmed by sequencing of Av1CF2 and included bases 28,596–30,471, the precise and complete deletion of Ad5 *Xba* I fragment D (Ginsberg, 1984). The incorporation of this deletion removes or disrupts all of the E3 genes with proven protein products (19, 14.7, 14.5, 11.6, 10.4, 6.7 kD) as well as one of the three unconfirmed open reading frames (7.5 kD, but not the 3.6 spliced kD or 12.5 kD; see Fig. 3 of Trapnell, 1993; Wold and Gooding, 1991).

The 293 cell line, which contains 11% of the left end of the Ad5 genome including functional copies of the E1 region genes, was used to produce high-titer stocks of all viruses and recombinant vectors as previously described (Rosenfeld *et al.*, 1992). Routinely, titers of these Av1 vectors of between 10^{11} and 10^{12} iu/ml were obtained as measured by plaque assay on 293 cells (Graham and Van Der Eb, 1973).

Correction of the CF epithelial cell phenotype

The ability of Av1CF2 to complement the abnormally regulated Cl^- permeability characteristic of CF epithelial cells was demonstrated in CFPAC cells (a cell line derived from an individual homozygous for the ΔF508 CFTR mutation) by measuring correction of short circuit I_{sc} abnormalities in a Ussing chamber (Fig. 2). After Av1CF2 infection, forskolin stimulation induced a large increase in Cl^- permeability as indicated by the rise in I_{sc} (Fig. 2A,D). As expected, uninfected CFPAC cells did not respond to forskolin-stimulation (Fig. 2B). As a further control, uninfected CFPAC cells were able to respond to ionomycin *via* an alternative, non-CFTR-dependent, Ca^{2+}-activated Cl^- channel, which is functional in cells from both CF patients and normals (Fig. 2C) (Anderson and Welsh, 1991). Similar to the results with CFPAC cells, MLE cells also showed an increased forskolin-stimulated I_{sc} compared to uninfected controls (Fig. 2E). Complementation of the CF epithelial cell Cl^- defect was also demonstrated by increased SPQ fluorescence indicating an increased forskolin-stimulated rate and extent of net Cl^- efflux in response to changes in Cl^-

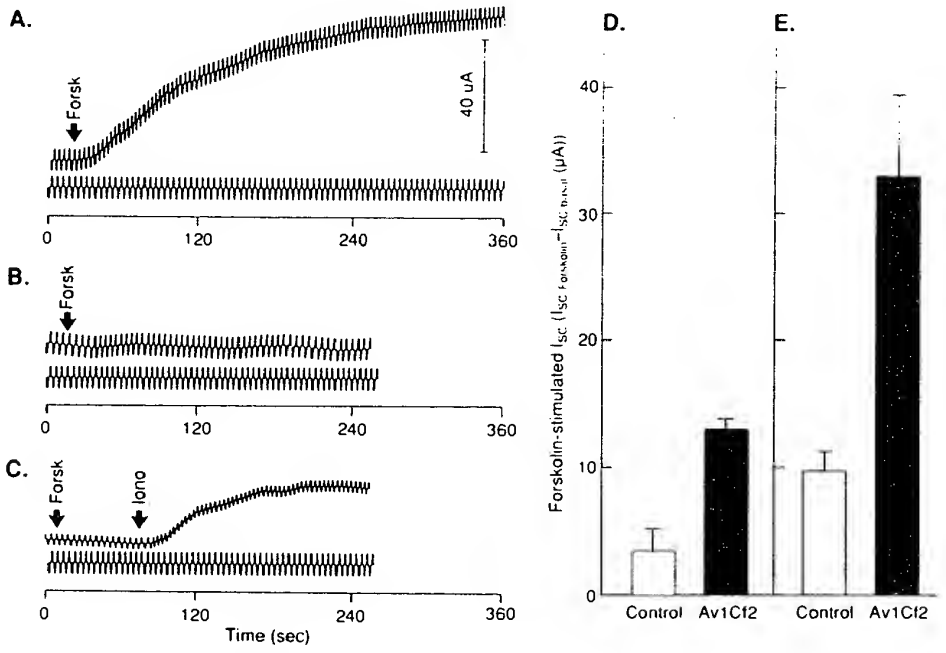

FIG. 2. Evaluation of Av1CF2-mediated complementation of the CF epithelial cell Cl^- secretory phenotype. CFPAC, a cell line from a homozygous $\Delta F508^-$ CF patient, or MLE cells, a murine lung epithelial cell line demonstrating the CF phenotype [absence of cAMP (forskolin)-stimulated Cl^- permeability] were grown on membrane supports, infected with Av1CF2 (50 iu/cell), and evaluated for forskolin-stimulated short circuit current 24 hr later. Based on studies with Av1LacZ4, an adenovirus reporter gene vector in a variety of lung cell lines, cellular transduction rates of approximately 50–80% are expected under these conditions. A. CFPAC cells infected with Av1CF2, demonstrating a rise in the short circuit current (I_{sc}) in CFPAC cells. B. Uninfected CFPAC cells showing no response to forskolin. C. Uninfected CFPAC cells again showing no response to forskolin, but demonstrating an increase in ionomycin (20 m*M* Iono)-stimulated I_{sc}. This is due to the presence of the Ca-activated chloride channel. D. CFPAC cells evaluated as above, showing the difference in forskolin-I_{sc} current from basal I_{sc}. I_{sc} measurements were obtained 4 min after forskolin addition. E. MLE cells evaluated as in D. In both D and E, data represent mean ± SEM for six separate determinations each.

content of the perfusion media (data not shown). Biological efficacy of Av1Cf2 was also confirmed by demonstrating expression of a specifically immunoprecipitable protein of the size expected for mature human CFTR and by measurements of $^{36}Cl^-$ efflux (Tolstoshev *et al.*, 1993) performed as previously described (Trapnell *et al.*, 1991b). This analysis establishes the functional expression of normal CFTR in CF patient epithelial cells, *in vitro*, through the use of the Av1Cf2 vector. The precise determination of the pharmacodynamics of Av1Cf2 in Cf cells is the subject of separate, future studies to be reported elsewhere.

Replication potential of Av1 vectors

One of the important safety concerns associated with clinical use of E1-deleted adenoviral vectors for *in vivo* human gene transfer relates to the defect in replication that is relevant to limiting pathogenicity and potential spread of the vector. Previous studies with E1-deleted adenoviral vectors have shown that the replication defect is a relative one which can be overcome in some cell type by increasing the moi and that at high moi, abundant production of infectious virions can occur (Shenk *et al.*, 1979). This is especially important, considering that some cell types express an E1a-like factor that can complement the replication defect (Imperiale *et al.*, 1984). Because of this concern, we have evaluated the capacity of Av1 vectors to replicate infectious virions as well as the relationship between moi and viral DNA synthesis. First, we infected HeLa cells, a human cell line permissive for human adenoviruses with Av1Cf1, Av1Null1, or as a positive control, the replication-competent adenovirus Ad-*dl*327 all at a moi of 60 iu/cell. The amount of infectious virions in cells or media was quantified by plaque assay on 293 cells at time points up to 3 days after infection (Fig. 3). As expected, Ad-*dl*327 replicated resulting in large increases of infectious virions. In contrast, neither Av1Cf1 nor Av1Null1 showed an increase in the amount of infectious virions, even after 3 days of incubation (Fig. 3). Thus, Av1 vectors do not replicate to high titers in this model system.

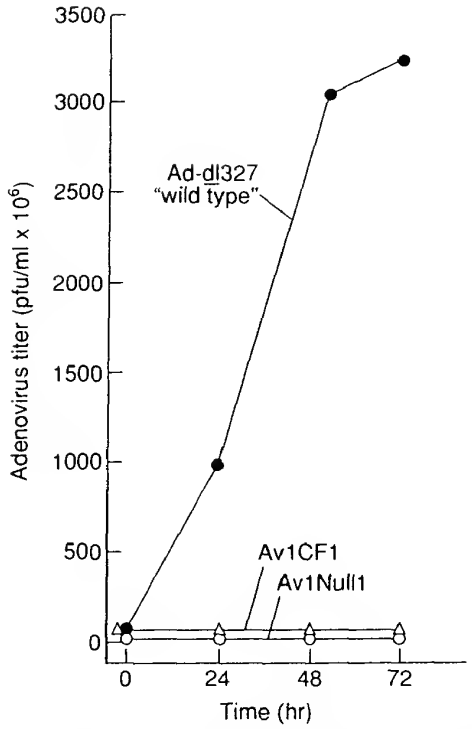

FIG. 3. Evaluation of the potential for production of infectious recombinant adenoviral vector particles from cultured epithelial cells infected by Av1 vectors or "wild-type" (replicative) virus (Ad-*dl*327). Ad-*dl*327, an E^- recombinant adenovirus with an intact E1 region harboring the same E3 deletion contained within the Av1 series vectors is fully replicative and is considered to be a wild-type adenovirus with respect to viral replication. As a positive control, this vector was able to replicate and produce infectious virus in HeLa cells. In marked contrast, neither Av1Null1, nor Av1Cf1 was able to replicate and produce infectious virions in these cells.

A second approach to the question of Av1 vector replication involved the sensitive technique of metabolic labeling of vector-infected cells with [^{32}P]orthophosphate followed by isolation of low-molecular-weight DNA (Hirt, 1967), restriction cleavage and electrophoretic analysis of labeled DNA fragments. This technique allowed the detection of metabolic labeling of any low-molecular-weight, episomal DNA (*e.g.*, adenoviral DNA), which becomes highly radioactively labeled (Berkner and Sharp, 1983), thus yielding diagnostic restriction cleavage fragments after autoradiography (Fig. 4). As expected, uninfected cells did not demonstrate labeled viral DNA bands (lane 1) whereas Ad5 clearly demonstrated characteristic labeled bands (lane 2), indicating that Ad5 was replicating in these cells. Importantly, at a moi of 30 iu/cell, Av1Cf1 did not show labeled DNA bands (lane 3). As a control, for the presence of Av1Cf1 DNA and to establish that replication-competent virus would be detected in the presence of vectors, co-infection of Av1Cf1 with Ad5 demonstrated complementation of the E1 defect and resulted in labeled DNA banding patterns characteristic for both Av1Cf1 and Ad5 (lane 4). This is undoubtedly due to complementation from Ad5 to overcome the $E1^-$ replication defect of Av1Cf1. However, it is interesting that Ad5 DNA appeared to replicate much less efficiently in the presence of vector (lane 4) than alone (compare lanes 2 and 4). A similar observation has previously been made for E3-deleted adenovirus mutants (Berkner and Sharp, 1983).

To evaluate the relationship of moi and viral DNA replication, HeLa cells were infected with Av1Cf2 at increasing moi values (10, 30, 100, 500, or 1,000 iu/cell) and evaluated for the presence of replicating viral DNA (Fig. 4B). No labeled bands characteristic of Av1Cf2 could be seen when the moi was less than 100 (lanes 5 and 6). However, at a moi of 100 iu/cell or more, very faint labeled bands characteristic of Av1Cf1 were visible if the autoradiograms were strongly overexposed (lanes 7–9). As a control, Ad5 easily demonstrated characteristic labeled DNA bands even at short exposure times (lane 10). Thus, HeLa cells supported replication of trace amounts of vector DNA although no infectious virus production was observed.

A third approach we have taken is to evaluate directly Av1 vector DNA replication in freshly isolated HBE cells infected with Av1Cf1. The results were reported elsewhere (Tolstoshev *et al.*,

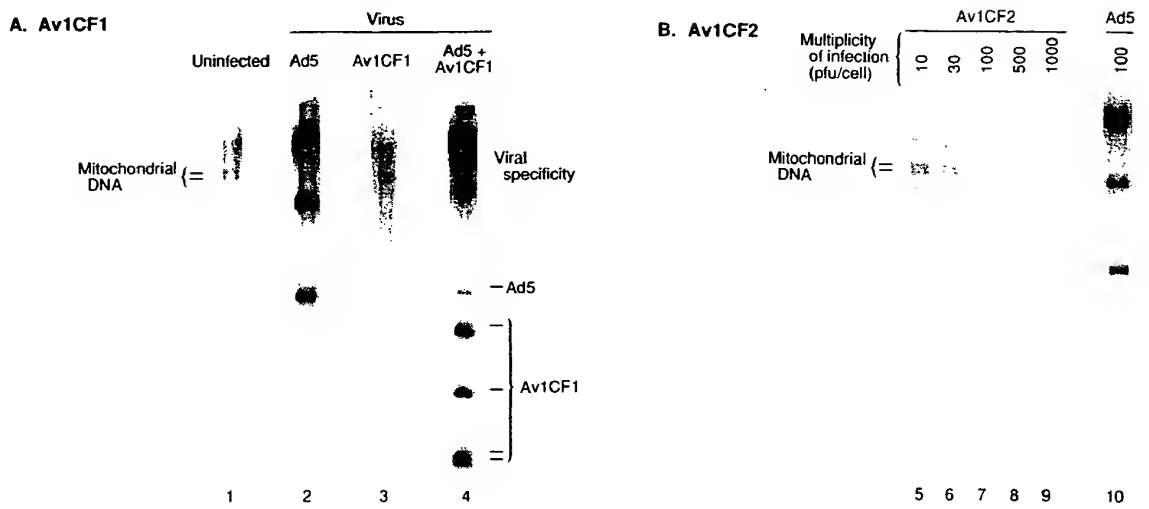

FIG. 4. Evaluation of the potential of Av1Cf vectors to replicate their viral genome in cultured epithelial cells. Metabolic labeling with ^{32}P-labeled nucleotides provides a sensitive assay for detecting viral DNA replication. A. Evaluation of Av1Cf1. Lane 1, as expected, no labeled viral DNA is seen in uninfected cells; lane 2, also as expected, Ad5 infection (moi = 30) resulted in strong labeling of viral DNA bands specific for Ad5; lane 3, in contrast, Av1Cf1 infection (moi = 30) resulted in no labeling of viral DNA; lane 4, when a small amount (moi = 3) of wild-type adenovirus was co-infected along with Av1Cf1 (moi = 30) so as to complement the E1-deficient vector, labeled viral DNA bands specific for both viruses could be seen. In all lanes, faint labeling of mitochondrial DNA was observed. B. Evaluation of Av1Cf2. Lanes 5–7, Av1Cf2 did not result in labeled viral DNA bands at moi values of 10, 30, and 100, even after long exposure of the autoradiogram; lanes 8 and 9, however, at moi values of 500 and 1000 with longer exposure of the autoradiogram, faintly labeled Av1Cf2 viral DNA bands were visible (difficult to see on the original overexposed film or to reproduce photographically); lane 10, as a positive control, parallel infection of cells with Ad5 resulted in strongly labeled viral DNA bands. In all lanes, faint labeling of mitochondrial DNA was observed.

1993), but showed no vector replication in the actual clinically relevant target cell even at a moi of up to 1,000 iu/cell.

Potential effects of Av1 vectors on the host cell genetic program

A second important concern regarding the use of defective recombinant adenoviral vectors that still contain structurally intact adenoviral genes is the potential expression of these genes and the possible toxic effects that their gene products might have on the cell. For example, some adenoviral proteins (*e.g.*, penton) are thought to be directly toxic (Ginsberg, 1984; Bai *et al.*, 1993). In this regard, morphologic evaluations were carried out in HeLa cells infected with Av1 vectors (Fig. 5). No cytopathic effect (CPE) was seen in uninfected cells (panel A) or cells infected with either Av1Null1 or Av1Cf2 (panels C and D, respectively). In contrast, cells infected with the replication-competent virus Ad-*dl*327 showed marked CPE (Bai *et al.*, 1993) with characteristic rounding of cells and loss of nuclear and nucleolar bodies (panel B).

The potential effects of Av1 vector on the host cell were further evaluated by examining the possible changes on the spectrum of host cell proteins synthesized in transduced and untransduced cells. The rationale for this derived from the knowledge that an important feature of wild-type adenovirus infection is the phenomenon of the shut-down of host cell protein synthesis mediated by E1b and E4 region gene products (Babbiss and Ginsberg, 1984; Pilder *et al.*, 1986; Dix and Leppard, 1993). To determine whether Av1 vectors retained or lost this function of host cell protein synthesis shutdown, total protein synthesis was evaluated by metabolic labeling of HeLa cells with [^{35}S]methionine followed by electrophoretic analysis of labeled proteins in whole-cell extracts (Fig. 6). Protein synthesis in uninfected cells (lane 1) was no different than in Av1Cf1-infected cells (lane 2) or Av1Cf2-infected cells (compare lanes 4 and 5). In contrast, wild-type adenovirus (Ad5) nearly completely shut down host cell protein synthesis (lane 3).

Similar experiments were conducted to evaluate the effects of Av1Cf1 vector infection on freshly isolated HBE cells, the actual cell target of the initial gene therapy strategies for CF. Further, to rule out a possible delayed effect, the studies were carried out over the course of 7 days (Fig. 7). The protein synthesis pattern of uninfected HBE cells (lanes 2, 6, 10, and 14) was no different than Av1Cf1-infected HBE cells (lanes 5, 9, 13, and 17). Similarly, Av1Null1 had no effect on host protein synthesis (lanes 4, 8, 12, and 16). In contrast, after Ad5 infection, host cell protein synthesis was diminished 1 day post infection and was shut off by 3 days post infection (lanes 3, 7, 11, and 15). Although host cell protein synthesis was reduced in wild-type virus infected cells, viral-specific proteins were still detectable by metabolically labeling at 7 days.

Expression of the adenovirus hexon gene

One of the mechanisms through which adenoviral vectors may induce pathology at the cellular level and through immune stimulation is the expression of adenoviral gene products, especially from the late genes (Bai *et al.*, 1993; Prince *et al.*, 1993;

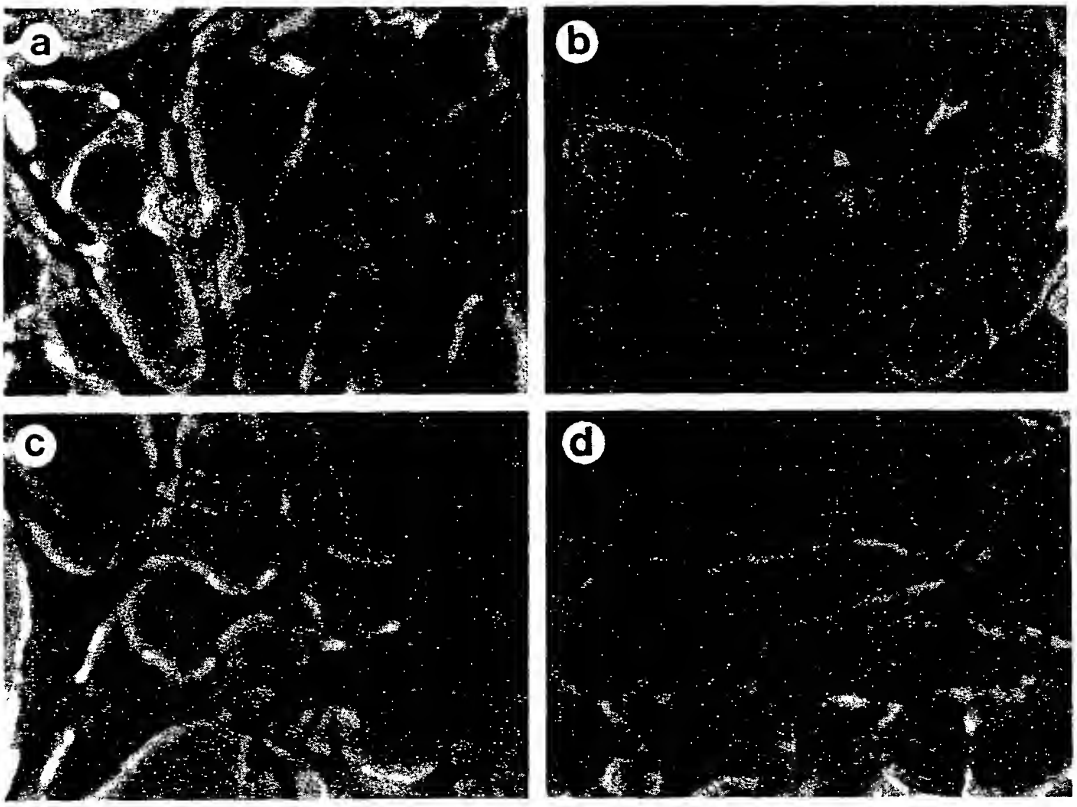

FIG. 5. Effect of Av1 vectors on cell morphology in HeLa cells after infection by various Av1 vectors or wild-type adenovirus. A. Untransduced HeLa cells. Note the concave polygonal cell shape with sharp, non-refractile cell and nuclear borders and prominent nucleoli (inverted phase, 600×). B. Similar to A, except 24 hr after Ad-*dl*327 transduction. Note the rounding of cells with loss of sharp cell and nuclear borders and nucleoli (inverted phase, 600×). C. Similar to A, except 24 hr after Av1Null1transduction. Note that there is no change in cell morphology compared to untransduced cells and cells still have concave polygonal cell shape with sharp, non-refractile cell and nuclear borders and prominent nucleoli (inverted phase, 600×). D. Similar to A, except 24 hr after Av1Cf1 transduction. Note that there is no change in cell morphology compared to untransduced cells and cells still have concave polygonal cell shape with sharp, non-refractile cell and nuclear borders and prominent nucleoli (inverted phase, 600×).

Rich *et al.*, 1993). Because of this, we examined expression of the adenoviral late gene encoding the hexon capsid protein (Fig. 8). Hexon is the most abundantly expressed adenoviral capsid protein in a wild-type adenovirus infection (Ginsberg, 1984; Horwitz, 1990b) and therefore is a good candidate for evaluation of late gene expression. As expected, hexon mRNA was abundantly expressed in 293 cells after infection by wild-type adenovirus (lane 4). Also as expected, no hexon mRNA was expressed in either uninfected 293 (lane 5) or HeLa (lane 6) cells. Interestingly, after a long exposure of the Northern blot, faint expression of hexon mRNA was observed in Av1Null1- (lane 7) and Av1Cf1- (lane 8) infected HeLa cells. Thus, at least in HeLa cells, the Av1 vector hexon gene could be expressed, albeit at very low levels compared to wild-type virus (compare lane 8 to lane 4). To evaluate whether hexon protein was also expressed, HeLa cells were evaluated by metabolic labeling of proteins with [^{35}S]methionine and immunoprecipitation with an anti-hexon antibody (Fig. 9). No hexon protein was observed in uninfected cells at any time during the 7 day period of evaluation. In contrast, hexon protein expression in Av1Cf2-infected cells was detected as early as 3 days post-infection. Expression peaked on day 4 and tapered thereafter, but was not absent at 7 days. Because equal amounts of total labeled proteins were loaded in each lane, the changes in expression reflect changes in hexon biosynthesis in proportion to that of cellular proteins. As a positive control, infection by the replication-competent vector Ad-*dl*327 resulted in abundant hexon protein expression as early as 1 day post infection.

In the context of the observations that: (i) a low level of replication of Av1 vectors may be occurring in HeLa cells; (ii) adenoviral late gene (*e.g.*, hexon) expression appears to be related temporally to replication (Horwitz, 1990b); and (iii) the hexon gene also appears to be expressed at a low level in HeLa cells, but no viral replication was observed in Av1Cf1-infected freshly isolated HBE cells even at a very high moi (*e.g.*, 1,000 iu/cell) (Tolstoshev *et al.*, 1993), we hypothesized that hexon gene expression might also be linked to replication in Av1 vectors. This concept was evaluated by examining hexon protein expression in freshly isolated HBE cells after infection by Av1Cf1 as described above (Fig. 10). Interestingly, hexon ex-

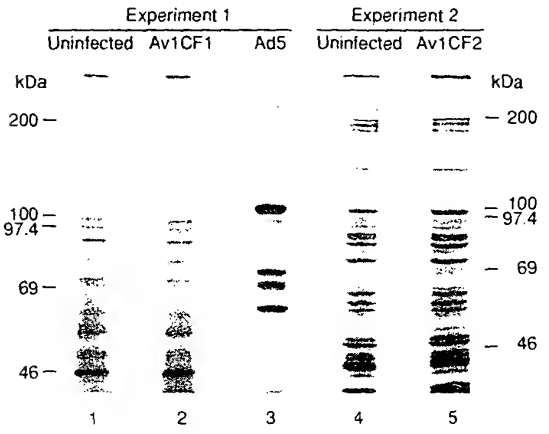

FIG. 6. Evaluation of the potential for Av1Cf vectors to alter the genetic program of host protein synthesis. Experiment 1. Lanes 1 and 2, The pattern of endogenous host cell protein synthesis was no different in uninfected cells versus cells infected by Av1Cf1; lane 3, in marked contrast, Ad5-infected cells showed a marked relative reduction in host cell protein synthesis with a replacement by adenoviral protein synthesis. The most abundant adenoviral protein was hexon, which can be seen at a position of approximately 110 kD. Experiment 2. Lanes 4 and 5, Similarly, Av1Cf2 fails to alter the pattern of endogenous, host cell protein synthesis compared to uninfected cells.

pression was not detected in HBE cells infected by Av1Cf1 (lane 4). As controls, abundant hexon expression was seen in Ad5-infected HBE cells (lane 3), but not in uninfected cells (lane 2). As a further control to demonstrate the presence of a functional hexon gene in the Av1 vector, 293 cells infected with Av1Cf1 or Ad5 demonstrated similar levels of hexon protein (lanes 7 and 6, respectively) while uninfected 293 cells did not (lane 5). Thus, Av1 vector-derived expression of the late gene for hexon capsid correlates with replication of viral DNA.

DISCUSSION

First-generation adenoviral vectors, rendered replication-deficient by deletion of the E1 region genes, are being evaluated for use in the treatment of the fatal pulmonary component of CF by transfer of the normal human CFTR cDNA (Crystal, 1992; Welsh, 1993; Wilson, 1993; Boucher and Knowles, 1993; Wilmott, *et al.*, 1993). In this study, we present data for one such vector, Av1Cf2, regarding preclinical, *in vitro* evaluation of biological efficacy and safety, and in an accompanying paper, data regarding *in vivo* safety evaluations (Yei *et al.*, 1994). Av1Cf2 is able to correct the Cl^- transport abnormalities in several cell lines, which demonstrate the $CFCl^-$ permeability phenotype. This is consistent with studies using similar, but not identical, vectors (Rosenfeld *et al.*, 1992; Engelhardt *et al.*, 1993; Rich *et al.*, 1993). Because the normal expression of CFTR in the lung is so low (Trapnell *et al.*, 1991a; Engelhardt *et al.*, 1993), estimation of the minimum amount of CFTR gene transfer and expression that will be required for therapeutic benefit is difficult to estimate. Interestingly, data for a similar vector, Ad2/CFTR-1, have shown a biologic effect at a moi as low as 0.1 iu/cell in CF nasal polyp epithelial cells *in vitro* (Rich *et al.*, 1993) and 1 iu/cell in nasal epithelium of CF

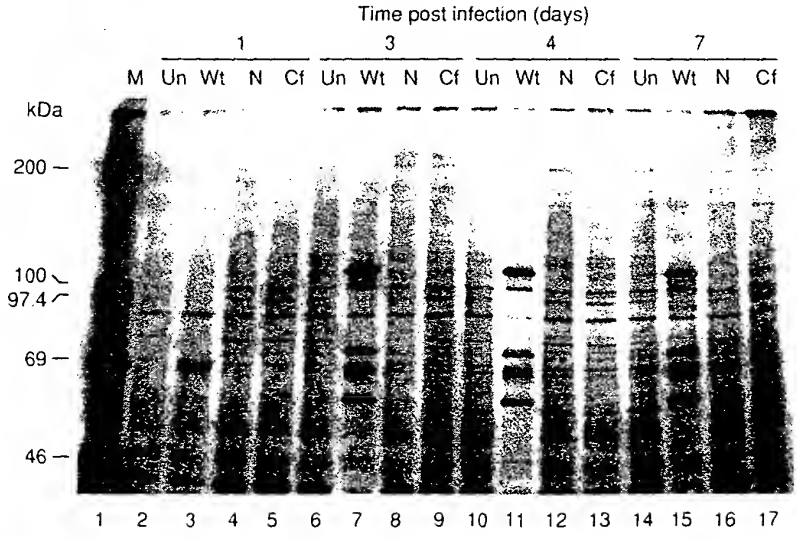

FIG. 7. Time course of the events after infection of human bronchial epithelial cells by wild-type adenovirus or one of several Av1 vectors. Lane 1, Protein molecular weight markers; lanes 2–5, 1 day after transduction; lanes 6–9, 3 days after transduction; lanes 10–13, 4 days after transduction; lanes 14–17, 7 days after transduction. Note at each time evaluated that compared to uninfected cells (Un), wild-type virus (W) infected cells have a marked alteration in the host cell protein synthesis pattern. At 1 day, this effect is partial, but by 3 days host cell protein synthesis is nearly completely shut down and replaced by viral protein synthesis. In contrast, cells transduced with Av1Null1 (N) or Av1Cf1 (Cf) show no change in the host cell protein synthesis pattern compared to uninfected cells.

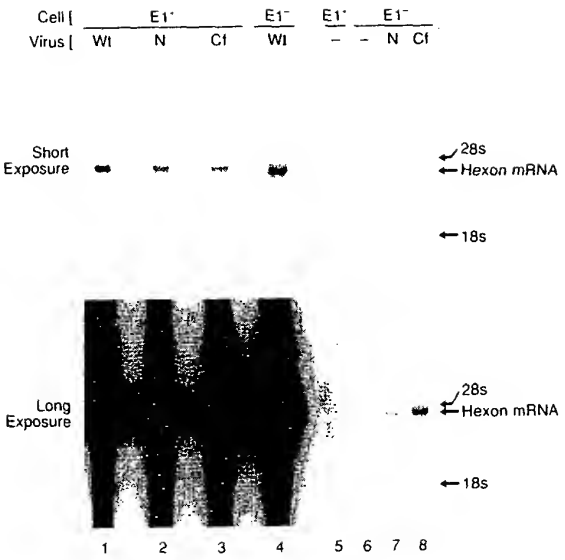

FIG. 8. Expression of mRNA transcripts for adenoviral hexon gene by wild-type human adenovirus and several Avl vectors in E1 complementing and non-E1 complementing cells. Short exposure. Lanes 1–3, In 293 cells that express E1 genes and are able to complement E1-defective adenoviral vectors, Ad5 hexon gene mRNA transcripts are expressed abundantly, as demonstrated by the prominent hybridizing bands of the expected size in cells infected by Ad5 (Wt, lane 1), Avl Null1 (N, lane 2), or Avl Cf1 (Cf, lane 3) after only a short exposure. Lane 4, Wild-type virus abundantly expresses hexon mRNA in non-E1 complementing cells (HeLa). Lanes 5 and 6, As expected, uninfected cells do not express adenoviral hexon mRNA transcripts. Lanes 7 and 8, At short exposure times, no hexon mRNA expression is detected in HeLa cells (which do not complement the E1 defect) infected with Avl Null1 or Avl Cf1. Long exposure lanes 1–4, As expected based on the short exposure, wild-type virus and vectors show abundant hexon mRNA expression in E1 complementing (293) cells, and wild-type virus does so also in non-E1 complementing (HeLa) cells. Lanes 5 and 6, Also as expected, the noninfected cells do not display hexon mRNA transcripts, even on prolonged exposure. Lanes 7 and 8, Interestingly, both Avl Null1 and Avl Cf1 showed a small amount of expression of hexon mRNA in non-E1 complementing cells after prolonged exposure of the autoradiogram. Importantly, the amount of Avl vector adenoviral hexon expression in non-E1 complementing cells is very much less than that in E1 complementing cells (compare lanes 2 and 7 and lanes 3 and 8) and is also very much less than the amount expressed in non-E1 complementing cells infected with a replicative wild-type virus such as Ad5 (compare lane 4 with lanes 7 and 8).

patients *in vivo* (Zabner *et al.*, 1993). This begins to define the theoretically possible lower end of the therapeutic window. For example, if one assumes that the epithelium of human airway contains 10^{10} cells, and that the surface epithelium of the airway is the appropriate clinical target, then this dose would be on the order of 10^9–10^{10} iu of the adenoviral vector.

The observation that Avl-type vectors do not produce measurable amounts of infectious virions after *in vitro* infection of HeLa cells supports the safety of these vectors with regard to the potential for horizontal transmission of the agent among treated patient contacts. This is consistent with prior observations which show that Avl Cf2 failed to replicate viral DNA in fresh human bronchial epithelial cells, even at a moi of up to 1,000 iu/cell (Tolstoshev *et al.*, 1993). The observation that at very high levels of moi, Avl vectors demonstrate trace levels of DNA replication in HeLa cells, but fail to produce detectable infectious virions, suggests that either other critical steps in Avl Cf2 virion production are blocked or that the production of infectious vector is so low as to be below the detection threshold of the plaque assay. The morphology of Avl vector-transduced cells also indicated a lack of replication of the vector. Although CPE is only a qualitative indicator in adenovirus-transduced cells, it is particularly useful in detecting the presence of wild-type, replicative adenovirus at extremely low levels. For example, CPE forms the basis of a critically important assay to detect potentially contaminating wild-type virus in vector preparations and is able to do so at a level of sensitivity of one replication-competent adenovirion per 10^7 cells even in the presence of as many as 10^8 replication-deficient vector virions (Trapnell, unpublished observations).

The finding that Avl vectors do not alter the morphology of infected cells suggests a lack of toxicity and further supports the safety of use of Avl Cf2. It is important to realize, that despite great utility of using CPE as an end point in detecting wild-type virus in cells when adenovirus gene expression overtakes that of the host cell, its use in the evaluation of cells transduced by nonreplicating vector may be limited by a threshold of expression of adenoviral genes when expressed at low levels, which occurs in some cells at least for the hexon gene. Although determination of this threshold is beyond the scope of our study, other methods were used to evaluate the effect of Avl vectors on the phenotype of the transduced cell. Specifically, Avl vector infection did not change the program of host cell protein synthesis observed in uninfected cells. This finding is especially important in view of the fact that wild-type adenovirus shuts off host cell protein synthesis nearly completely (Ginsburg, 1984). Genes located within E1b and E4 regions are thought to mediate this shut-down. The adenoviral gene products thought to mediate this process include the 55-kD E1b protein (Babbiss and Ginsberg, 1984; Pilder *et al.*, 1986) and the E4 open reading frame (ORF) 6 (Dix and Leppard, 1993). The absence of the shutdown phenomenon in Avl vectors [which are unable to code for the 55-kD E1b protein (Trapnell, 1993) but do encode a structurally intact E4 ORF gene product] is consistent with a lack of expression of the latter or a requirement of interaction of the two proteins for the shutdown phenomenon, as has been suggested (Dix and Leppard, 1993).

Adenoviral hexon gene expression by Avl vectors occurs in HeLa cells *in vitro*, albeit in trace amounts very far below wild-type levels and not at all in freshly isolated HBE cells. These observations are consistent with the observed pattern of DNA synthesis in these two cell types and supports the concept that the transition from early to late gene expression is associated with events occurring at the time of adenoviral DNA synthesis (Ginsberg, 1984; Horwitz 1990b). The lack of Avl vector-derived gene expression in HBE cells, the target of current clinical trials, is important, but the observation of hexon expression in HeLa cells *in vitro* suggests a possible mechanism

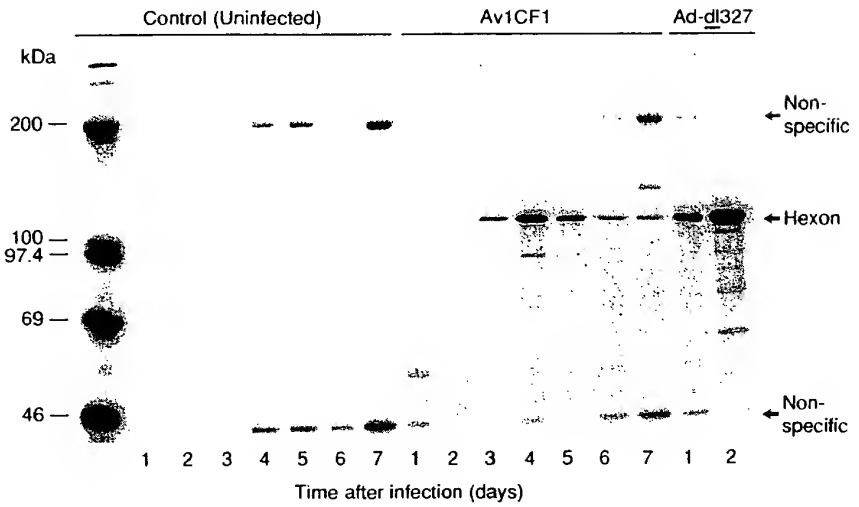

FIG. 9. Expression of adenoviral hexon gene protein by wild-type human adenovirus and several Av1 vectors in HeLa cells. As a control, no adenoviral hexon protein could be demonstrated in uninfected cells for up to 7 days of the experiment. In contrast, after infection of HeLa cells with Av1Cf1, adenoviral hexon protein was present on days 3 through 7 with a peak in expression at day 4 and a decline thereafter. No expression was seen in Av1Cf1-infected cells on days 1 and 2 after infection. In contrast, the fully replicative virus, Ad-*dl*327, showed significantly higher hexon expression on days 1 and 2.

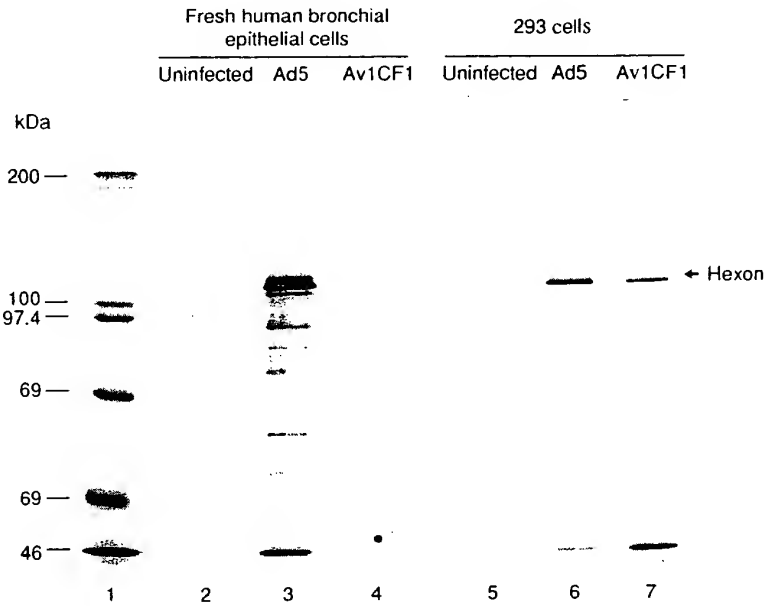

FIG. 10. Expression of adenoviral hexon gene protein by wild-type human adenovirus, but not by Av1Cf1, in freshly isolated human bronchial epithelial cells. Lane 1, Molecular weight markers of the indicated sizes; lane 2, no adenovirus hexon protein could be immuno-precipitated from uninfected control cells; lane 3, as expected, Ad5 infection of HBE resulted in abundant expression of immunoprecipitable adenoviral hexon protein; lane 4, importantly, no adenoviral hexon protein could be demonstrated in Av1Cf1-infected HBE cells, even with the use of the sensitive technique of specific immuno-precipitation. This is consistent with the very small amount of hexon mRNA expression in Av1Cf1 vector infected non-E1 complementing cells compared to that of wild-type virus. Lanes 5–7, As controls for vector viability, infection, and function, hexon was not seen in uninfected 293 cells but was easily demonstrated in Ad5- and Av1Cf1-infected cells.

for host-immune system removal of infected cells on the basis of cell-mediated immunity. This finding has important implications for the use of these vectors in human clinical trials, possibly in relation to the pharmacokinetics of the gene therapy with regard to the duration of the expression of adenovirus-derived normal CFTR.

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